

## FOUR PORTRAITS OF THE PLAGUE ORGANISM

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*Yersinia pestis* organisms have infective and self-protective features that are expressed or suppressed to fit different conditions. The *Y. pestis* bacteria respond selectively to a variety of signals (which occur inside or outside of diverse hosts and cells) by expressing products that ultimately promote the multiplication and survival of the organisms. This paper briefly outlines the dynamics and time courses of the warfare between invading bacteria and susceptible hosts. At least four different sets of products and mechanisms are described as characteristic of natural life cycles of wild-type *Y. pestis* in non-human hosts. This information is used to project likely hazards for human beings, design of effective vaccines, and selection of other countermeasures.

### INTRODUCTION

This paper resulted from a requirement to estimate human hazard levels (and probable consequences) of feasible military attacks involving weaponized *Y. pestis* organisms<sup>1</sup>. Such requirements pose severe problems. In such cases, experimental data collection is impractical. Experimental methods would require unavailable human subjects, inappropriate use of animals, or unreasonable resources. Computer-aided modeling offers alternatives, but (as with other modeling) usefulness depends upon effective projection of realities in the field of interest. Modeling of biological warfare hazards involves realities of organism or toxin characteristics, and their interactions with live environments. Since potential enemies do not share data on characteristics of weaponized *Y. pestis* organisms, it was necessary to base estimates on parameters of (and experience with) wild-type *Y. pestis*. Accordingly, it became necessary to project consequences from human inhalation of an organism adapted for success in promoting infections of fleas and rodents. This necessity led to extrapolations of conclusions drawn by plague specialists. Such interpretations were strongly dependent upon perspectives provided in two comprehensive reviews by Dr. Robert Brubaker<sup>2,3</sup> and one by Drs. Robert Perry and Jacqueline Fetherston<sup>4</sup>. In lieu of extensive citations, references to these reviews are implied or used wherever possible, below. A few citations are provided indirectly as references listed in modeling reports<sup>1,5</sup>. In some cases these indirect modeling references are localized to one of 40 numbered paragraphs (ex.:<sup>1-40</sup>) or a section (ex.:<sup>5-4.1</sup>) of the cited report. Papers by Dr. Joseph Hinnebusch, *et al.*<sup>6-8</sup> illustrate flea anatomy and relationships of *Y. pestis* properties to pathophysiology in fleas. Photographs of a blocked flea and *Y. pestis* in tissue have been related to biological warfare by Drs. Thomas McGovern and George Christopher<sup>9</sup>. Initial capitals are used, in this paper, to distinguish protein products from their genes (ex.: Ymt vs. *ymt*).

<b>Report Documentation Page</b>			<i>Form Approved OMB No. 0704-0188</i>		
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1. REPORT DATE <b>00 JAN 2002</b>	2. REPORT TYPE <b>N/A</b>	3. DATES COVERED <b>-</b>			
4. TITLE AND SUBTITLE  <b>Four Portraits Of The Plague Organism</b>			5a. CONTRACT NUMBER		
			5b. GRANT NUMBER		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S)			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) <b>Science Applications International Corporation Biomedical Modeling and Analysis Program</b>			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)			10. SPONSOR/MONITOR'S ACRONYM(S)		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION/AVAILABILITY STATEMENT <b>Approved for public release, distribution unlimited</b>					
13. SUPPLEMENTARY NOTES <b>This article is from ADA409494 Proceedings of the 2001 ECBC Scientific Conference on Chemical and Biological Defense Research, 6-8 March , Marriott's Hunt Valley Inn, Hunt Valley, MD.</b>					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT <b>UU</b>	18. NUMBER OF PAGES <b>7</b>	19a. NAME OF RESPONSIBLE PERSON
a. REPORT <b>unclassified</b>	b. ABSTRACT <b>unclassified</b>	c. THIS PAGE <b>unclassified</b>			

## *Y. pestis* in the flea digestive tract $\sim 25^{\circ}\text{C}$

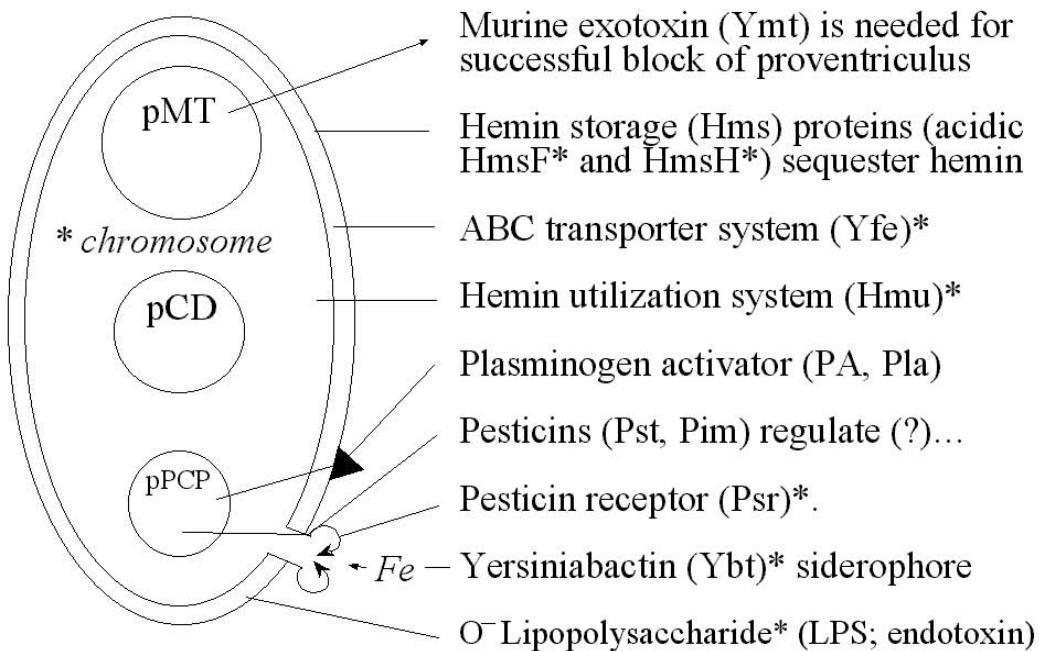


Figure 1. The virulence of *Yersinia pestis* bacteria has been associated with proteins encoded by genes in chromosomal DNA and three plasmids. As represented above, the pMT1 (murine toxin) plasmid is the largest. The mid-sized pCD1 (calcium dependence) plasmid is not expressed in fleas. Although it is small, the pPCP (pesticin, coagulase, plasminogen activator) plasmid is active in both fleas and their hosts. Hinnebusch et al.<sup>8</sup> report that Ymt exotoxin is primarily expressed at flea temperatures, and is important for survival of *Y. pestis* (for transmission via the flea) but it is not required to kill mice. Perry<sup>10</sup> observes that “Pathogens must acquire iron from the iron-deficient environment of their mammalian hosts to survive and cause disease...the siderophore-dependent yersiniabactin (Ybt) iron transport system is required during the early stages of plague infections. An independent Yfe iron transport system functions later in the infectious process to acquire the iron essential for growth. [Another *Y. pestis* system allows use of] heme-containing proteins...a temperature-regulated hemin-storage [Hms system] absorbs hemin to the outer membrane...and is required for transmission of the disease from fleas to mammals.” Hinnebusch et al.<sup>7</sup> found that Hms proteins are required for blockage of the flea proventriculus with *Y. pestis* that are regurgitated into flea bite wounds in hosts. They concluded that sequestered hemin of *Y. pestis* outer membranes causes them to be “...extremely aggregative and hydrophobic.” Such bacteria stick to each other sufficiently to ‘gum up’ spines of the proventriculus a few days after infection occurs in the flea midgut<sup>8</sup>. This action was shown as independent of the plasminogen activator (Pla). Pla is now believed to aid host uptake of bacteria from a bite wound, rather than affecting the flea<sup>8</sup>. It appears that some of the *Y. pestis* iron-acquiring mechanisms are also expressed in the flea primarily in readiness for use in a host. This may be true for LPS, Yfe, Hmu, and the pesticin receptor (Psn) system, including pesticin (Psn), its inhibitor (Pim), and Ybt.

## *Y. pestis* arrives passively in wound & lung

Status in flea becomes status in host, pending temperature effects

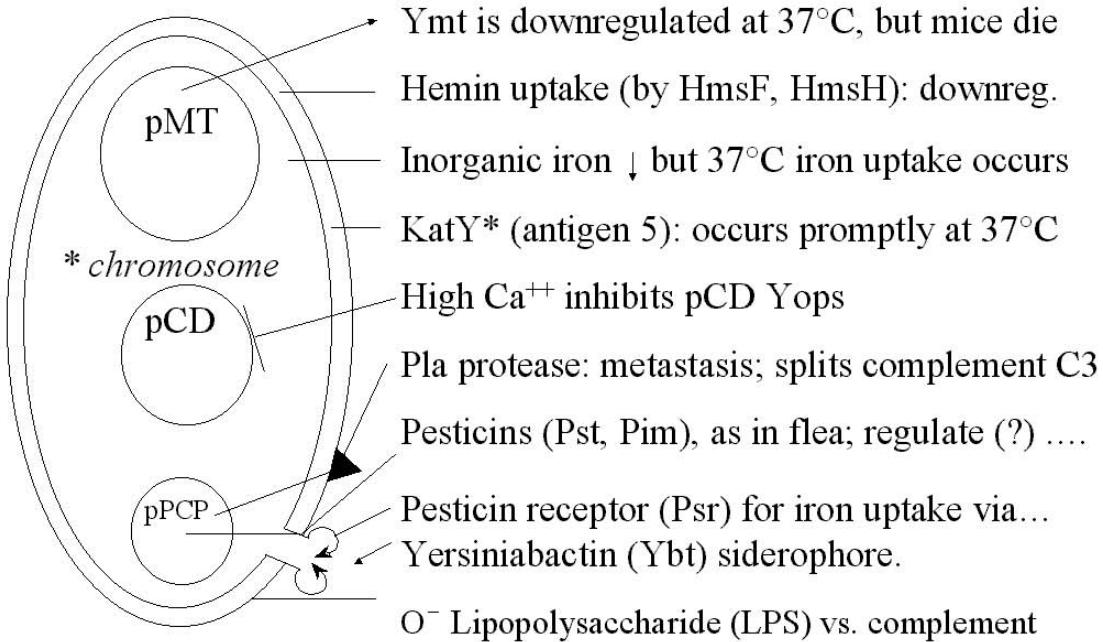


Figure 2. Continued Ymt exotoxin export may be useful but not necessary at 37°C. However, the key to *Y. pestis* success in warm bodies is temporary vulnerability<sup>1-27</sup>. *Y. pestis* present as a harmless Trojan horse to be taken inside host macrophages, which are then sabotaged. This stage may last for 1/4 hrs<sup>1-9,24</sup>. Perhaps only the bacteria prepared for survival in a macrophage are relevant for infection; Finegold<sup>1</sup> found only about 100 *Y. pestis*/lung at 12-16 hrs after an inhalation exposure. Prepared bacteria will have features primarily needed in the flea, but specific products of iron storage may have a brief role. Already stored hemin may be needed for *Y. pestis* survival of destruction from H<sub>2</sub>O<sub>2</sub> in the macrophage<sup>1-32</sup>; the KatY in periplasm<sup>3</sup> may provide a second line of catalase defense. Extracellular blockade of pCD prevents an attack by *Y. pestis* before they are safely within the macrophage. Pre-existing Pla is ready to lyse clots or membranes that might isolate extracellular *Y. pestis* from local macrophage intake, or transfer to lymph nodes. Pla protects *Y. pestis* from phagocyte chemotaxis<sup>1-9</sup> or opsonization<sup>1-14</sup> by C3 complement protein in blood. The unornamented (O<sup>-</sup>, sidechain free) LPS<sup>1-6</sup> of *Y. pestis* also prevents placement of the complement membrane attack complex. It appears that some iron-related processes are temperature specific to meet changing conditions. It is less clear why and when given systems are needed. After mentioning the Ybt, Yfe, and Hmu systems, Perry<sup>10</sup> states, "All three of these uptake systems are expressed only when needed and we are examining the regulatory mechanisms which control their expression." It is known that Psr binds both Pst and Ybt. Iron delivered to Psr by Ybt is handled by a cytoplasmic iron transfer protein known as TonB, under control of a feedback regulator known as Fur<sup>1-30</sup>. This iron transfer system is found in other *Y. pestis* iron uptake systems<sup>4</sup>. Brubaker<sup>2</sup> observed that Psn may aid iron uptake, but lack of Psn does not reduce virulence of *Y. pestis* in the presence of iron. Psn kills Psn-deficient mutants. Brubaker<sup>11</sup> suggests that Psn kills *Y. pestis* mutants that might arouse effective host immune responses. However, Psn might induce toxic levels of iron if not regulated by Pim. This would explain Psn killing of bacteria without Pim.

## Transformation in macrophage ~ pH6

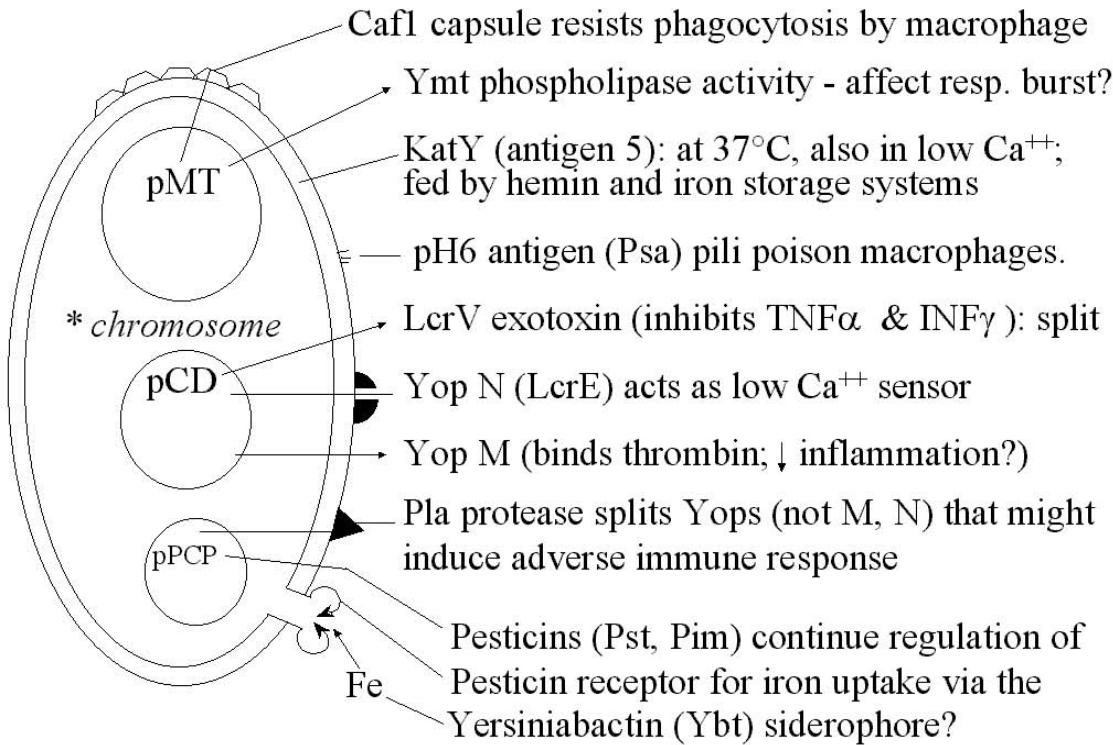


Figure 3. Caf1 (F1 capsular antigen of Plague Vaccine, USP<sup>1-7</sup>) is freely expressed by *Y. pestis* in cultures at 37°C, but probably not before macrophage uptake *in vivo*<sup>1-27</sup>. However, Davis et. al.<sup>4</sup> have shown that capsules do not impede virulence of cultured *Y. pestis* inhaled into monkey lungs. This is important because acidic alveolar macrophage lysosomes may have relatively weak oxidative bursts<sup>1-26</sup>, making them easy targets for *Y. pestis* to parasitize. The Ymt phospholipase activity inhibits cell respiration in mice<sup>1-7</sup>. Such activity and/or suppression (Bauldry<sup>1</sup>) of tumor necrosis factor (TNF $\alpha$ ) may depress phagocyte respiratory bursts. Such suppression could be slow (or hemin insufficient) to neutralize lysosomal H<sub>2</sub>O<sub>2</sub>. Rapid deployment of more catalase (Fe+KatY) may provide defense-in-depth<sup>1-32</sup>. *Y. pestis* not only survive but adapt, multiply and escape from macrophages<sup>1-31</sup>. They adapt to very acidic conditions (pH 4.6-4.8<sup>1-34</sup>) within the lysosome by expressing fibers (pili) of pH 6 antigen (Psa). Pili are cytotoxic to macrophages, preventing antigen presentation or antibody formation<sup>1-34</sup>. Further suppression of host immune defenses is made potentially possible by the activation of transcription factor LcrH (under intracellular conditions) to express some *Yersinia* outer proteins (Yops)<sup>2,3,4</sup>. However, LcrV inside cells is destroyed by Pla protease. Presumably LcrE is needed, and LcrV and Yop M become exotoxins, only after the multiplied *Y. pestis* burst from macrophages. As in the flea, the *Y. pestis* bacteria must simultaneously flourish in their current intracellular environment and be preparing for transition to the next. Although handling of iron is critical (more than 10% of *Y. pestis* membrane protein is involved with this function<sup>1-28</sup>), it is not clear how iron handling takes place within the macrophage. One guess is that internal status directs change. Hemin retention and KatY may dominate while *Y. pestis* must resist oxidation under acid conditions in the lysosome. When the lysosome is disabled, the multiplying *Y. pestis* may use other systems to reprocess iron stored by the initial organism. Then the bacteria may use Ybt to scavenge iron from macrophage cytoplasm poisoned by pili. Finally, systems must be in place for survival during extracellular transit (in lymph or blood) to nodes, liver, or spleen.

## Extracellular contact at $\text{Ca}^{++}$ sensor

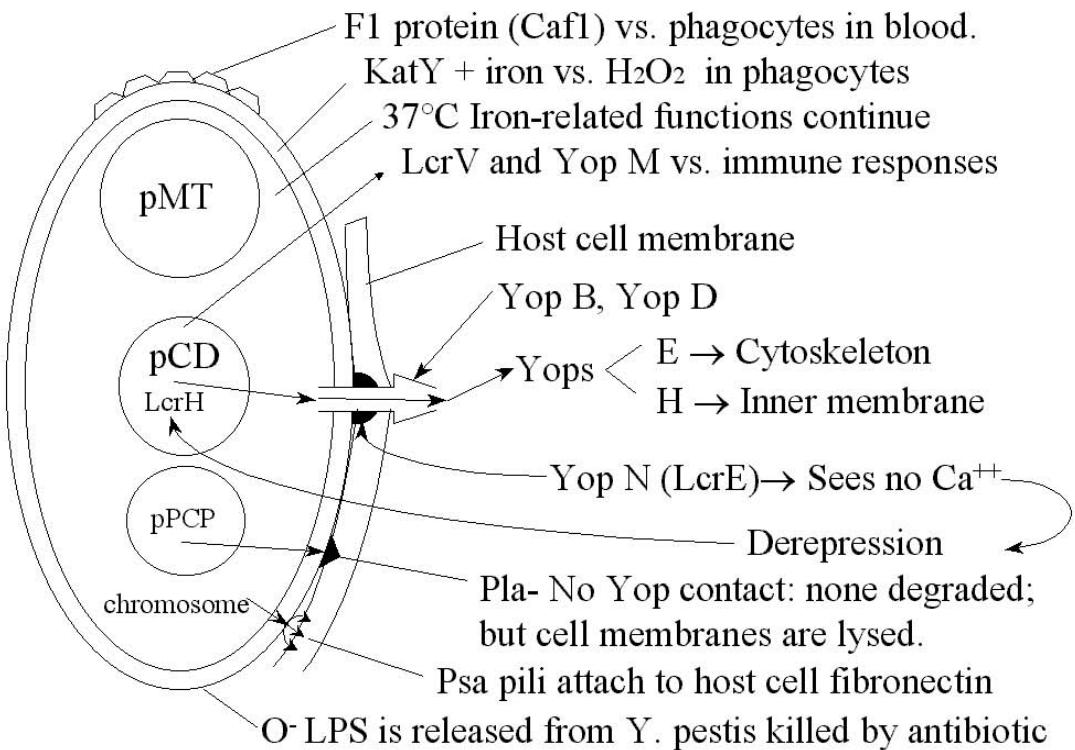


Figure 4. In macrophages, synthesis of Psa was detected after 90 min. and pili after 4hrs, but F1 capsular antigen caught up after 18 hrs<sup>1-33</sup>. Assembled Caf1 subunits<sup>1-7</sup> largely shield pili in culture. This may represent the extracellular state in which Caf1 repels phagocytosis. KatY is expressed abundantly at 37°C, and it may scavenge  $\text{H}_2\text{O}_2$  in foci made necrotic by *Y. pestis*<sup>3</sup>, but virulence is unaffected by deletion of genes for KatY<sup>11</sup> or Caf1<sup>1,3</sup>. The diverse iron-acquisition system assures uptake of the iron required for massive extracellular multiplication of *Y. pestis*<sup>4</sup>. LcrV is expressed independently of cytotoxic Yops to serve as an exotoxin that suppresses usual immune responses to foreign protein or bacteria<sup>3,4</sup>. LcrV does this by blocking upregulation of interferon gamma (IFN $\gamma$ ) and tumor necrosis factor alpha (TNF $\alpha$ ). As noted by Brubaker<sup>4</sup>, these cytokines "...fill indispensable roles in non-specifically activating professional phagocytes and mediating formation of protective granulomas...[*Y. pestis*] maintains the illusion that the host has no cause for alarm. In short, plague is a disease of stealth." Yop M helps to maintain that illusion by interfering with inflammatory responses involving thrombin and platelets. It has been suggested that this blocks mediators of chemotaxis, histamine release, vascular permeability, and migration of leukocytes<sup>1-16</sup>. *Y. pestis* use Psa and a type III secretory pathway<sup>2</sup> as follows: "...while employing pili to pin their opponents, at close quarters the invaders deploy syringes and dispatch host cells by injecting cytotoxic Yops. Gaining strength from captured nutritional assets...the invading masses cause focal necrosis and...terminal levels of bacteremia...with endotoxin shock.<sup>1</sup>" The roles of Yops B, D, E and H (and other cytotoxins) in this process (and external disabling of phagocytes) have been illustrated in more detail elsewhere<sup>2,4</sup>. However, it should be noted that Psa pili permit *Y. pestis* to adhere both to fibronectin of host membranes and mucin of mucous membranes<sup>1-36</sup>. LcrE needs close contact to exclude extracellular  $\text{Ca}^{++}$ . Pla binds collagen<sup>2</sup> and "...promotes adherence<sup>4</sup>..." until its proteolytic action opens membranes. According to Finegold et al.<sup>1</sup>, LPS may be under-rated as a cause of death from *Y. pestis*. Others have observed that antibiotics may kill *Y. pestis*, then their LPS kills with hemorrhagic shock<sup>1</sup>.

TABLE 1. Relative Impacts of Conceivable Vaccine Antigens on *Y. pestis* virulence.

Antigens	Attenuation Factor*	References
Pgm (pigmentation: usage of iron)	10 <sup>8</sup>	Kutyrev, <i>et al.</i> (1989, 1992) <sup>4</sup>
Lcr (transcription of all Yops)	10 <sup>7</sup>	Une & Brubaker (1984) <sup>4</sup>
LcrV (V exotoxin)	10 <sup>7</sup>	Brubaker (1996) <sup>1</sup>
pPCP (pesticins + Pla)	10 <sup>7</sup>	Brubaker, <i>et al.</i> (1965) <sup>1</sup>
Pla (plasminogen activator)	10 <sup>6</sup>	Sodinde <i>et al.</i> (1992) <sup>4</sup>
Yops E, H, KL or Lcr (cytotoxicity)	10 <sup>6</sup>	Straley & Bowmer (1986) <sup>4</sup>
Yop M (binds thrombin)	10 <sup>5</sup>	Leung <i>et al.</i> (1990) <sup>4</sup>
pH6 (Psa) antigen (pili)	10 <sup>4</sup>	Lindler <i>et al.</i> (1990) <sup>4</sup>
Yop D (cytotoxicity)	10 <sup>3</sup>	Andrews <i>et al.</i> (1999) <sup>3</sup>
Yop N (LcrE; Ca <sup>++</sup> sensor)	1	Andrews <i>et al.</i> (1999) <sup>3</sup>
Caf1 (F1 protein)	1	Pitt, <i>et al.</i> (1990) <sup>1</sup>
Kat Y (catalase)	1	Brubaker (2001) <sup>11</sup>
Psr (pesticin receptor)	?	-
O <sup>-</sup> Lipopolysaccharide (endotoxin)	?	-

\* Approximated attenuation factors given as *Y. pestis* log dose reduction with antigen deleted, rounded to nearest log; studies may not be completely comparable.

Table 1. A substantial portion of the human population does not develop effective antibodies to a given vaccine. Accordingly, trivalent vaccines (such as the annual influenza vaccine) are used in hope that seroconversion failure will not occur with all antigens. This prompted thoughts of a future trivalent plague vaccine<sup>5</sup>. Kutyrev *et al.* (1989)<sup>4</sup> list the minimal requirements for *Y. pestis* pathogenicity as functioning Pgm and LcrV. This idea is supported by successful long term Russian use of live *Y. pestis* in human vaccination programs<sup>1,5</sup>. Such vaccination relies on absence of the multiple Pgm genes from *Y. pestis* strain EV-76. Brubaker<sup>3</sup> has reported apparent success of experimental vaccines that incorporate LcrV as a subunit antigen. He also mentioned hearsay of Russian success with a vaccine using Pla as the antigen<sup>5-3,5</sup>. This table might suggest that targeting of Yops could be effective. However, antibodies need access to an antigen displayed on the bacterial surface, like capsular antigen of Plague Vaccine USP. Figures 3 and 4 show that most Yops are expressed when and where they are not readily accessible to antibodies. As secreted exotoxins, LcrV is (and Yop M may be) accessible. LcrE is the only other Yop reported as resistant to proteolysis by Pla. The report of Andrews *et al.*<sup>4</sup> suggests that *Y. pestis* has alternatives to use of LcrE for activation of the type III secretory pathway. Vodop'ianov *et al.* (1995)<sup>5</sup> found that cytotoxic effects of Psa pili prevented attempts to induce antibodies to this surface-displayed antigen. It appears that a surface-displayed member of the Pgm family might be a promising candidate. KatY is primarily located in periplasm and otherwise unpromising<sup>4,11</sup>. This leaves the pesticin receptor as a candidate for evaluation. It remains to be seen whether components of other iron-related systems are available to antibodies. However, it is possible that antigen accessibility should be assessed for *Y. pestis* in the form considered in Figure 4. A review<sup>1</sup> of human pneumonic plague transmission suggests that this is the form of *Y. pestis* that was inhaled. Russian investigators<sup>5-3,6</sup> claim success with vaccination by inhalation of dry *Y. pestis* strain EV-76. Butler (1996)<sup>5</sup> has developed monoclonal antibody anti-endotoxins. It is not clear that O<sup>-</sup> LPS, as displayed on the surface of *Y. pestis*, has been tried in a vaccine.

## SUMMARY

The plague organism presents in at least four distinctively different forms, depending upon its current environment. Since biological warfare is likely to involve inhalation exposures<sup>9</sup>, it follows that a future anti-plague vaccine should be optimized to prevent pulmonary macrophage access by either passive or pneumonic forms of *Y. pestis*. Presently available data suggest that a future inhalable trivalent vaccine might beneficially include subunit antigens for LcrV, Pla, and Psr and/or other iron-related *Y. pestis* antigens.

## ACKNOWLEDGEMENTS

This paper is a result of research (under Contract No. DAMD17-93-C-3141) directed by COL Gerald Parker, (then) Deputy for Medical, Chemical, and Biological Defense, U.S. Army Medical Research and Materiel Command, Fort Detrick, MD 21702-5012, with initial project management by (then) MAJ George Korch and subsequent support by (then) LTC Kelly Davis.

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